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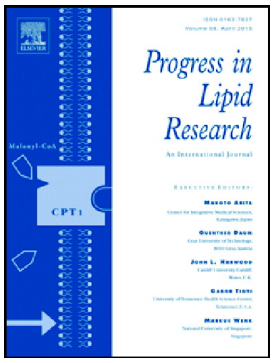

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PII: S0163-7827(17)30037-1
DOI: doi: 10.1016/j.plipres.2017.09.002
Reference: JPLR 948

To appear in: *Progress in Lipid Research*

Received date: 8 June 2017
Revised date: 29 August 2017
Accepted date: 6 September 2017

Please cite this article as: Bo Yang, He Gao, Catherine Stanton, R. Paul Ross, Hao Zhang, Yong Q. Chen, Haiqin Chen, Wei Chen , Bacterial conjugated linoleic acid production and their applications. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Jplr(2017), doi: 10.1016/j.plipres.2017.09.002

Bacterial conjugated linoleic acid production and their applications

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ABSTRACTS

Conjugated linoleic acid (CLA) has been shown to exert various potential physiological properties including anti-carcinogenic, anti-obesity, anti-cardiovascular and anti-diabetic activities, and consequently has been considered as a promising food supplement. Bacterial biosynthesis of CLA is an attractive approach for commercial production due to its high isomer-selectivity and convenient purification process. Many bacterial species have been reported to convert free linoleic acid (LA) to CLA, hitherto only the precise CLA-producing mechanisms in *Propionibacterium acnes* and *Lactobacillus plantarum* have been illustrated completely, prompting the development of recombinant technology used in CLA production. The purpose of the article is to review the bacterial CLA producers as well as the recent progress on describing the mechanism of microbial CLA-production. Furthermore, the advances and potential in the heterologous expression of CLA genetic determinants will be presented.

Key words: conjugated linoleic acid, bacterial CLA-producers, CLA bioconversion mechanism, microbial cell factories

1. Introduction

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid (C18:2, *c*9,*c*12) with conjugated double bonds. CLA has attracted great interest in recent decades due to its biological and physiological benefits [1, 2], including anti-carcinogenesis [3-5], anti-obesity [6-9], anti-inflammation [10, 11], anti-diabetic [12-14] as well as bone formation-promoting properties [15, 16]. Many bacteria have been reported to convert free LA into CLA, such as *Butyrivibrio fibrisolvens* [17], *Lactobacillus reuteri* [18], *L. plantarum* [19, 20], *L. casei* [21], *L. acidophilus* [22], *Bifidobacterium breve* [23], *B. longum* [24], *Propionibacterium acnes* [25], *P. freudenreichii* [26] and *Clostridium sporogenes* [27].

Most commercial CLA is produced via chemical isomerization of LA, however, the chemical process will result in unexpected by-products, which are mostly harmful [28]. Although CLA has different isomers, many studies have confirmed that only *c*9,*t*11-CLA, *t*9,*t*11-CLA and *t*10,*c*12-CLA are considered as the isomers with beneficial activities. Considering the usage of CLA for medical purposes, a safe isomer-selective process is required. Microbial CLA seems to be a perfect approach, although the CLA concentration typically produced during microbial fermentation is insufficient for commercial purposes. A potentially better alternative is the use of genetically modified organism (GMO) recombineered to overproduce CLA with high efficiency. The advances in the identification of CLA-producing mechanisms among some strains have prompted the development of recombinant technology in CLA production such as *Escherichia coli* [29] and *Yarrowia lipolytica* [30, 31].

This article will review the current knowledge on microbial CLA producers and the precise mechanisms for bacterial CLA production, as well as recombinant DNA technology for industrial CLA production.

2. Bacterial CLA producers

According to the major CLA isomers, the strains that can produce CLA can be divided into two groups: 9,11-CLA (*c*9,*t*11-CLA and *t*9,*t*11-CLA) and *t*10,*c*12-CLA. Some typical strains with high conversion rates are listed in Table 1.

2.1 *c*9,*t*11-CLA and *t*9,*t*11-CLA producers

2.1.1 Rumen bacteria

Rumen bacteria can bio-hydrogenate some unsaturated fatty acids to *trans*-vaccenic acid

or stearic acid with CLA as intermediate [32]. Since demonstration of the capacity of *B. fibrisolvens* A38 to produce CLA efficiently among rumen bacteria, it has been widely used as a model for CLA production in rumen bacteria [33-35]. It is reported that 40% of LA is converted to CLA by this strain, of which 95% is the *c*9,*t*11-CLA. Furthermore, accumulation of CLA is inhibited by such factors as high concentration of LA [36], aerobic conditions [36] as well as glycolytic inhibitors [37]. CLA reductase activity has been identified as another important factor influencing CLA production. For example, *B. fibrisolvens* TH1 exhibits high CLA isomerase activity, but the accumulation of CLA is low, mostly due to high reductase activity, catalyzing the conversion of CLA into *trans*-vaccenic acid [38]. In contrast, the accumulation of CLA occurs in *B. fibrisolvens* MDT-5 due to the absence of CLA reductase activity. Thus, the latter strain could be considered to be an ideal probiotic for animal nutrition for its high CLA production capability [39].

2.1.2 *Lactobacillus*

Lactobacilli have attracted more attention than other CLA-producing strains due to their health-promoting effects. Many species of lactic acid bacteria have been reported to possess the ability to produce CLA. *L. reuteri* was the first species of lactic acid bacteria reported with high CLA production capability [40]. Subsequently, Lee et al. [41] found that immobilized *L. reuteri* cells could accumulate 5.5 times more CLA than that obtained from the conversion by free washed cells. Furthermore, glycocholate which occurs in humans is shown not to influence CLA production by *L. reuteri* ATCC 55739 [42]. The bioconversion capability of CLA in *L. reuteri* at different conditions was investigated [43], and highest concentration of CLA was obtained in broth containing 20 mg/l free LA aerobically at 10 ^{o}C for 30 h. *L. plantarum* is the most widely reported species among lactobacilli with high CLA-production ability. In 2002, Kishino et al. identified some strains that have the ability to generate CLA, in which *L. plantarum* AKU1009a showed the highest conversion rate, with up to 85% of LA being converted into *c*9,*t*11-CLA [19]. CLA could also be accumulated at a level of up to 2700 mg/l by *L. plantarum* JCM1551 with ricinoleic acid (12-hydroxy-*cis*-9-octadecenoic acid) as the substrate [44]. It is also reported that both the growing culture and washed cells of *L. plantarum* ZS2058 could effectively convert LA into CLA, at rates of 54.3% for growing cultures and 46.75% for washed cells, respectively [45]. Additionally, washed cells and substrate

concentration, manipulation of the content of yeast extract and glucose in MRS broth increased the CLA productivity significantly in *L. plantarum* [46]. Linoleic acid could be converted into different hydroxyl fatty acids such as 10-HOE, 10,13-diHOA, and 13-hydroxy-*cis*-9-ocatadecenoic acid (13-HOE). For instance, *L. reuteri* LTH 2584 [47], *L. sanfranciscensis* ATCC27651[48, 49], *L. hammesii* DSM16381 [47-49], *L. spicheri* LS38 [47], *L. rhamnosus* LGG [50], *L. plantarum* ST-III [50], *L. acidophilus* NCFM [50], *L. plantarum* AKU1009a [19, 51-55], *L. plantarum* ATCC 8014 [56], *L. plantarum* ZS2058 [45] and *L. acidophilus* AKU1137 [28, 57] could generate 10-HOE. In the CLA producers, 10-HOE was accumulated during CLA production in *L. acidophilus* AKU1137, *L. plantaurm* AKU1009a and, *L. plantarum* ZS2058 and *L. plantarum* ATCC8014, and was confirmed as CLA-generation intermediates. In hence, the enzyme catalyzing 10-HOE production should be essential for CLA production in those strains. *L. acidophilus* NBRC 13951 [58] could convert LA to 10-HOE and 13-HOE, while *L. plantarum* TMW 1.460 [47, 48], *L. acidophilus* LMG 11470 [59, 60], and *L. acidophilus* AKU1137 [61] could metabolize LA into three hydroxyl fatty acid including 10-HOE, 13-HOE,10,13-diHOA. Heretofore, no evidence shows 13-HOE or 10,13-diHOA are involved in CLA production.

Washed cells of *L. acidophilus* La-5 accumulated CLA in the cells, but not in the culture medium. In addition, absence of oxygen could only influence the ratio of different CLA isomers produced, but not total CLA concentration. Tween 80 has been shown to be effective to promote growth and *c*9,*t*11-CLA production efficiency of *L. acidophilus* F0221 in the presence of bile salts [62]. Similar to *L. acidophilus*, immobilized cells of *L. delbrueckii* subsp. *bulgaricus* onto polyacrylamide could significantly increase the content of CLA compared with that by free washed cells [63]. Other *Lactobacillus* strains have shown high abilities to produce CLA, such as *L. casei*, *L. pentosus* and *L. brevis* [19]. The application of *L. casei* for the synthesis of CLA in both hen eggs and broiler meat cuts were demonstrated, suggesting this strain as a suitable probiotic for such application [64].

2.1.3 *Bifidobacterium*

The ability of *Bifidobacterium* to produce CLA was firstly reported by Coakley and colleagues [65], in which nine of fifteen strains presented high CLA-producing activity with *c*9,*t*11-CLA as the predominant isomer produced and members of *B. breve* species as the

most efficient for CLA production. One hundred and fifty strains of bifidobacteria strains were isolated from human intestines [66], and four isolates showed 80% conversion of LA to CLA in MRS broth. Among these strains, *B. breve* LMC 017 could convert up to 90% of linoleic acid or 78.8% of monolinolein into CLA. *B. breve* LMC520 is reported to produce CLA with maximal bioconversion rate up to 90% [23]. Thirty six bifidobacteria strains were assessed for CLA production [67] and consequently four *B. breve strains* were discovered to transform LA into CLA ranging from 19.5% to 53.5%. More than 70% of CLA isomers produced by *B. breve* were *c*9,*t*11-CLA, meanwhile approximately 38% of CLA isomers were *t*9,*t*11-CLA in *B. breve* LMG 13194. *B. longum* is another high CLA production species among the genus *Bifidobacerium*. Rapid screening of CLA-producing bifidobacteria was established by Barrett et al. [24]. With this method, four *B. longum* strains, isolated from feces, were found to convert more than 20% of free LA to CLA [24]. *B. longum* DPC6320, demonstrated 43.89% *c*9,*t*11-CLA conversion, while *B. longum* DPC6315 could convert only 11.02% of free LA into *c*9,*t*11-CLA [68]. Another study showed that *B. longum* could increase the content of CLA in the cheese by 20.44% [69]. *B. animalis* Bb12, one of the most widely used probiotics, could transfer 27% of free LA into *c*9,*t*11-CLA in MRS broth [65]. *B. animalis* BLC showed the best CLA production with free LA as substrate while *B. animalis* Bb12-1 demonstrated the highest conversion rate of CLA with ricinoleic acid as substrate [70]. Additionally, *B. dentium* NCFB 2243 could convert 29% of LA into 9,11-CLA [47]. The CLA conversion rate of *B. bifidum* CRL 1399 was up to 24.8% in MRS broth [71]. Gorrisen and colleagues [67] discovered that *B. bifidum* LMG 10645 could produce CLA from LA with conversion rate of 40.7%. *B. animalis* subsp.*lactis* Bb-12 [50], *B. breve* NCIMB 702258 [72] could produce 10-HOE during CLA generation.

2.1.4 *Propionibacterium*

The first CLA-production *Propionibacteria* was reported by Verhulst and colleagues [26], in which *P. freudenreichii* subsp. *freudenreicbii*, *P. freudenreichii* subsp. *shermanii*, *P. acidi-propionici* and *P. tecbnicum* could produce *c*9,*t*11-CLA. Jiang et al. [73] analyzed dairy starter cultures for the capability to produce CLA from free LA in MRS broth. Three *Propionibacteria* strains were discovered to generate CLA with high efficiency. *P. freudenreichii* subsp. *freudenreichii* Propioni-6 Wiesby showed the highest CLA-production capability (35.3% conversion). *P. shermanii* AKU1254 could produce 0.11 g/l CLA in reaction mixture with 4 g/l

free LA, and the CLA produced was the mixture of *c*9,*t*11-CLA and *t*9,*t*11-CLA [19].

2.1.5 *Clostridium*

Several strains of *Clostridium bifermentans*, *C. sporogenes* and *C. sordelli* were shown to hydrogenate LA into *trans*-vaccenic acid *in vitro* with *c*9,*t*11-CLA as intermediate [27]. Peng et al. [74] showed that *c*9,*t*11-CLA accumulated in *C. sporogenes* ATCC 22762 within 30 min and then *t*9,*t*11-CLA and *t*10,*t*12-CLA increased at the expense of *c*9,*t*11-CLA until these reached the same level.

2.1.6 Other *c*9,*t*11-CLA producers

Other strains showed the ability to produce CLA. *Lactococcus lactis* subsp. *cremoris* CCRC12586, *L. lactis* subsp. *lactis* CCRC 10791 and *S. thermophilus* CCRC 12257 were reported to convert free linoleic acid in skim milk plus 12% free linoleic acid [22]. Additionally, some other *Lactococcus* [75], *Streptococcus* [76], *Leuconostoc* and *Pediococcus* [76, 77] strains have shown the capability to produce CLA with different substrates.

2.2 *t*10,*c*12-CLA producers

Bacterial species which could produce *t*10,*c*12-CLA are less common than those producing *c*9,*t*11-CLA. *T*10,*c*12-CLA is the isomer presenting significant benefits on anti-obesity [78]. *P. acnes* has been shown to convert LA into *t*10,*c*12-CLA. Verhulst and colleagues firstly reported the ability of *P. acnes* to produce *t*10,*c*12-CLA, including strain ATCC 6919, ATCC 6921, VP1 162, VPI 163, VPI 164, VPI 174, VPI 186, and VPI 199 [26]. *P. acnes* ATCC 6919, could also catalyze the production of *t*10,*c*12-CLA from linoleic acid at high level [79].

Additionally, *t*10,*c*12-CLA could be also produced from linoleic acid by *Megasphaera elsdenii* [36]. *M. elsdenii* YJ-4 could catalyze 35% of free LA into CLA, among which the percentage of *t*10,*c*12-CLA is up to 85% [80] . Peng et al.[74] showed that *C. sporogenes* ATCC 22762 could generate *t*10,*t*12-CLA at a low level while *c*9,*t*11-CLA was the major isomer produced.

While *c*9,*t*11-CLA was the major isomer in lactic acid bacteria, *t*10,*c*12-CLA isomer could be produced by some lactic acid bacteria strains [21]. Enzymes were purified from *Lactobacillus* strains to determine their capability for CLA production and consequently, *L. rhamnosus* PL60 and *L. pentosus* IFO 12011 were identified to produce considerable

*t*10,*c*12-CLA isomer [81]. CLA produced by *L. plantarum* NCUL005 consists of 32.2% *c*9,*t*11-CLA isomer and 67.8% *t*10,*c*12-CLA isomers [82]. In *L. reuteri* ATCC55739, *t*10,*c*12-CLA could make up 41% of the final CLA isomers [18]. As well as *P. freudenreichii* with high CLA production ability could accumulate a smaller amount of *t*10,*c*12-CLA when they produce amount of *c*9,*t*11-CLA and *t*9,*t*11-CLA from linoleic acid [73].

3. Role of CLA production in bacteria

The role of conjugated fatty acids in bacterial cells is unclear, but a number of proposals have been made. For example, it has been proposed that bio-hydrogenation is a means for anaerobic bacteria to dispose of reducing power [83]. Bacterial cells capable of isomerizing linoleic acid to CLA exhibit greater tolerance to linoleic acid, compared with bacteria that exhibit non-CLA production. In a research by Jiang et al [73], it was reported that the majority of CLA-producing *Propionibacteria* were those inhibited by the presence of high concentrations of free linoleic acid, and a positive correlation between microbial CLA production and tolerance to linoleic acid was observed among the CLA producing strains. This suggested that microbial CLA production may be a detoxification mechanism for the bacterial cell. Linoleic acid has been shown to be toxic to many bacteria, as shown by lack of ability to grow in the presence of the fatty acid [21, 73, 84, 85]. When cultured in the presence of the more rigid CLA molecule, as compared with linoleic acid, bacterial cells exhibited superior growth, while growth was unaffected by stearic acid [85]. In general, long chain fatty acids with a higher degree of unsaturation are reportedly more inhibitory bacterial cell growth than fatty acids of the same chain length but with fewer unsaturated double bonds [86]. The presence of linoleic acid and CLA in the medium has been shown to up-regulate the molecular chaperone *GroEL* in *B. fibrisolvens* by 6.9 and 5.5-fold, respectively [87]. This induced expression of *GroEL* is believed to be a non-specific response to stress rather than a specific mechanism enabling *B. fibrisolvens* to withstand the toxic effects of linoleic acid and CLA.

4. Bacterial CLA-production mechanism

A number of species and strains generating CLA were reported, however, heretofore mechanisms for CLA bioconversion have not been elucidated for each species.

4.1 Mechanism for *c*9,*t*11-CLA production

4.1.1 Rumen bacteria

Two processes involved in the bioconversion of LA into stearic acid exist in the mixed rumen bacteria. The first process is from LA to monoenoic acid, followed by the process from monoenoic acid to stearic acid [88, 89]. The enzyme which catalyzes the conversion of linoleic acid to *c*9,*t*11-CLA is linoleate isomerase. The isomerase from *B. fibrisolvens* is reported to be membrane-bound and have maximum activity within a narrow substrate concentration range (α-linolenic aicd and γ-linolenic acid), and to have absolute specificity for a substrate containing a free carboxyl group and a *c*9,*c*12 diene bonding system. Cofactors seem to be not essential for the isomerase because enzymatic activity would not be influenced by passing through corresponding chromatographic columns or the addition of CoA, ATP, Mg^{2+}, ADP, AMP and NAD^{+} [35]. Isomeration of LA into CLA by the crude enzyme extracts was not affected by the aerobic condition, even though anaerobic condition was essential for intact cells [89, 90]. The preferred ω (omega) chain length was shown to be 6 carbons, as heptadecadienoic acid (C17:2, ω7) was isomerized at only half the rate of LA [35, 91]. Kepler et al. [91] proposed a model for the isomerization of LA by linoleate isomerase, in which the substrate binds initially to the enzyme in a hydrophobic pocket. The binding involves interaction of the π-electrons of the substrate double bond with an electrophilic group on the enzyme and hydrogen bonding of the undissociated carboxyl group of the substrate with an electronegative center in the enzyme (Fig. 1) [92]. It was concluded that the hydrogen added to carbon 13 was derived from water, which suggests that the hydration added to carbon 13 was derived from water, which suggests that the hydration-dehydration mechanism generates ricinoleic acid as an intermediate, although no hydroxyl fatty acids were detected.

Hydrogenation of CLA by reductase activity could influence the amount of the CLA in these strains. CLA reductase is approximately 60 kDa, which could convert the CLA to the *t*9 or *t*11 ocatdecenoic acid [93, 94]. Fukuda et al. demonstrated that addition of saturated fatty acid could stimulate the activity of isomerase, while the activity of CLA reductase would increase with the addition of unsaturated fatty acid [95]. Fukuda et al. also discovers that *cla-r* is responsible for the isomerase activity [39, 93]. Though many properties of the isomerase have been clarified, the purification of linoleate isomerase from the *B. fibriosolvens* is still unsuccessful. More effort should be taken to obtain the crystal structure of isomerase in order to clarify the essence of the isomerization.

4.1.2 *Lactobacillus*

Putative linoleate isomerase in lactobacilli is considered as the key factor involved in the bioconversion of LA into CLA, similar to that in the rumen bacteria and *Propionibacterium*, in which no intermediates were accumulated. Rosson et al. isolated and characterized a special enzyme from *L. reuteri* ATCC55739, which was originally identified as isomerase [96], and the size of which was reported as 67 kD with an optimum pH of 6.8-7.5 [97]. Moreover, this protein is identified to be homogenous to myosin-cross-reactive antigen (MCRA), which is universal among bacteria. However, when the gene was cloned into various expression systems, no CLA was produced, although recombinant *Bacillus subtilis* produced a fatty acid with the same molecular weight as a hydroxylated linoleic acid derivative at the expense of linoleic acid [97]. High homology between the purified linoleate isomerase from *L. reuteri* and the relationship between hydroxyl fatty acid and CLA have already been discovered. Volkov et al. firstly demonstrated that MCRA from *S. pyogenes* is a fatty acid hydratase not a linoleate isomerase [98]. In addition, the crystal structure of MCRA from *L. acidophilus* NCFM was analysed [99]. FAD was found to be loosely linked to MCRA. Furthermore, four intricately connected domains were observed in the crystal structure, and three domains (domain1, domain2 and domain 3) are responsible for the formation of a hydrophobic substrate channel, similar to that found in several flavin-dependent amino-oxidases (Fig. 2). With linoleic acid as substrate, 10-hydroxy-*cis*-12-octadecenoic acid (10-HOE) and 10,13-dihydroxy-octadecanoic acid (10,13-diHOA) were both produced by the purified MCRA from *L. acidophilus* NCFM. MCRA is successfully purified from *L. plantarum* AKU1009a [51-55, 100], identified as FAD-independent hydratase with NADH as the activator, similar to other MCRAs [50, 98]. It is also reported in this study that $FADH_2$, produced by hydrating FAD by NADH, may be the actual activator for the reaction. The hydratase seems to preferentially act with the substrate coupled with ethylenic bond in the hydration reaction, such as the linoleic acid, oleic acid and that with hydroxyl at Δ10 position in the dehydration reaction. The result is similar to that of MCRA isolated from *Elizabethkingia meningoseptica* [101], *S. pyogenes* [98], and *B. breve* [72]. Further studies performed in our lab demonstrate that MCRA in different lactic acid bacteria functioned as hydratase not a linoleate isomerase [50]. Similar results were obtained in other researches [102, 103]. Therefore, the mechanism for CLA production in lactobacilli remained

unclear.

Multiple-step reactions for CLA production was hypothesized and several intermediates were determined respectively. Ogawa et al. [28] reported that following incubation with washed cells of various cultures; two different isomers of CLA were detected (*c*9,*t*11-CLA and *t*9,*t*11-CLA) together with two hydroxyl fatty acids, 10-hydroxy-*trans*-12-octadecenoic acid and 10-HOE. To elucidate the mechanism of CLA production, *L. acidophilus* AKU 1137 was found to accumulate hydroxyl fatty acids before CLA production, which decreased rapidly as the reaction proceeded (Fig. 3). This finding suggests that the two hydroxyl fatty acids are intermediates in CLA production, starting with hydration of linoleic acid to 10-hydroxy-octadecenoic acid and subsequent dehydrating isomerization of the hydroxyl fatty acid to CLA. Furthermore, hydroxyl fatty acids when used as substrate were converted to CLA by *L. acidophilus*. Free ricinoleic acid was a substrate for CLA production for a wide range of LAB [104]. In 2011, Kishino et al. successfully isolated three proteins from *L. plantarum* AKU1009a, in which one was membrane-protein involved in the forming 10-hydroxyl-*cis*-12-octadecenic acid from LA and the other two proteins existed in the cytoplasm. Together with these three proteins, LA could be converted to CLA [52], and those proteins are highly homogenous to MCRA, short-chain dehydrogenase/oxidoreductase and acetoacetate decarboxylase of *L. plantarum* WCFS1, respectively. Based on the N-terminal sequencing, three genes involved in the production of CLA from linoleic acid, *cla-hy, cla-dh and cla-dc*, were identified [51]. For further analysis, the three genes were heterologously expressed in *E. coli* and with the mixture of the recombinants CLA was generated successfully, which was the first report for the confirmed mechanism of CLA production in lactic acid bacteria. Later research in the group, the whole metabolism of LA was illustrated; with hydroxyl fatty acids and oxo fatty acids as intermediates, LA could be converted into CLA and oleic acid, those metabolites were catalyzed by CLA-HY (10-LAH), CLA-DH, CLA-DC, and CLA-ER [53]. This pathway includes the process of hydration, dehydration and double-bond immigration, and the precise pathway was described as follows (Fig. 3). According to position of hydroxyl group, the enzymes catalyzing linoleic acid hydration could be divided into two groups: linoleate 10-hydratase (10-LAH) and linoleate 13-hydratase (13-LAH), in which 10-LAH is defined as oleate hydratase (EC 4.2.1.53). Interestedly, the enzymes converting LA to hydroxyl

fatty acids are belonged to MCRA family, which means MCRA is multifunctional. MCRA from *L. planarum* AKU1009a [51-55], *L. plantarum* ZS2058 [45], *L. plantarum* ATCC8014 [56], *L. reuteri* LTH2584 [47], *L. hammessi* DSM16381 [47], *L. rhamnosus* LGG [50] and *L. plantarum* ST-III [50] were cloned and identified as FAD-dependent linoleate 10-hydrase, in which FAD was confirmed as an essential factor. Several linoleate 13-hydtastases were characterized as well. MCRA from *L. acidophilus* NCFM was characterized [50], and its crystal structure was analyzed, with the purified protein 10-HOE and 10,13-diHOA could be produced from LA [99]. In addition, MCRA of *L. acidophilus* LMG 11470 was characterized with multiple functions as well which could convert LA to 10-HOE and 13-HOE [59]. Current knowledge on the linoleate 13-hydratase shows that they are not FAD-dependent. However, how 10-LAH and 13-LAH generated 10,13-diHOA remains unclear.

Similar results are observed in our lab in *L. plantarum* ZS2058, a strain with efficient production of CLA. 10-hydroxyl-*cis*-12-octadeneic acid, 10-oxo-*cis*-12-octadeneic acid, 10-oxo-*trans*-octadeneic acid are detected, which is considered as the intermediates during the isomerization of LA into CLA. Moreover, the corresponding genes were also identified in detail [45].

A more recent publication reported that 10-HOE could be transferred into *c*9,*t*11-CLA directly by enolase (Fig. 3) [105]. This novel enzyme could not recognize LA as the substrate instead 10-HOE. These results enriches our understanding of producing CLA among *L. plantarum*, which indicated multiple or alternative mechanisms for CLA production in lactobacilli.

4.1.3 *Bifidobacterium*

The information about mechanism of CLA production in *Bifidobacterium* is limited. Rosberg-Cody et al. [72] firstly reported a putative linoleate isomerase gene in *B. breve*, which is highly homogenous to *mcra* in *L. reuteri* ATCC 55739. This corresponding enzyme is identified as FAD-dependent protein, catalyzing reaction from the LA to 10-hydroxy-*cis*-12-octadecacenic acid, similar to our finding in *B. lactis* Bb12 [50]. It's reasonable to speculate that 10-hydoxy-*cis*-12-octadeceneic acid might be the first intermediate in bifidobacterial CLA generation. However, after *mcra* was knocked out, MCRA in the *B. breve* has been reported to be only active for oleate hydrogenation nor CLA

316 production in the *mcra* knock-out mutant [106]. The mechanism for CLA production in
317 *Bifidobacterium* remains unclear, which needs further investigation.

318 4.1.4 *Clostridium*

319 Linoleic acid could be isomerized by *C. sporogenes* to *c*9,*t*11-CLA [27], followed by
320 accumulation of *t*9,*t*11-CLA and *t*10,*t*12-CLA isomers at the expense of *c*9,*t*11-CLA isomers
321 [74]. *C. sporogenes* linoleate isomerase has already been purified, identified to be
322 membrane-associated and shown to be unstable, especially being solubilized by detergents.
323 Similar to the substrate specificity of isomerase in *B. fibrisolvens*, *P. acnes* and *L. plantarum*,
324 *cis* double bond at the *c*9 and *c*12 position of C18 polyunsaturated fatty acids with free
325 carboxyl group seems to be necessary in *C. sporogenes.* Furthermore, no external cofactors
326 or energy sources were required for isomerization. However, the corresponding gene(s) has
327 not been identified yet and detailed characterization of this enzyme is still unclear.

328 **4.2 Mechanism for *t*10,*c*12-CLA production**

329 In contrast to linoleate isomerase responsible for production of *c*9,*t*11-CLA in lactic acid
330 bacteria, polyunsaturated fatty acid isomerase (PAI) involved in the bioconversion of LA to
331 *t*10,*c*12-CLA in *P. acnes* has been clearly elucidated [79, 107, 108]. Bioconversion of CLA by *P.*
332 *acnes* is confirmed to be one-step and nonredox isomeration, in which linoleic acid is directly
333 catalyzed into *t*10,*c*12-CLA [79] . PAI is also identified to be FAD-dependent, however, with no
334 requirement for reducing power (NADH or NADPH) and also to be soluble protein with 424
335 amino acid, different from those isolated from *B. fibrisolvens* [17], *L. plantarum* [51] and *C.*
336 *sporogenes* [74]. Furthermore, crystal structure of PAI has already been elucidated, in which
337 PAI and PAI-LA complexes are purified and then determined (Fig. 4) [108, 109]. Three
338 intricately connected domains are found in PAI, in which FAD could be nonvalently linked to
339 domain 1 and positively charged patches generated by several Lys and Arg residues could be
340 localized near the channel entrance in domain 3 serving as an initial recognition site for the
341 carboxylate group of the fatty acid. It has been noted that isomerization of LA by PAI is initiated
342 by abstracting a hydrogen anion at C11 directly toward the atom N15 of FAD and then
343 carbocation is produced, served as the intermediate stabilized by π-cation and helix dipole.
344 *T*10,*c*12-CLA is then generated by addition of the hydrogen at C9 and reproduction of FAD.

345 The PAI represents the first report of a crystal structure reported for a fatty acid isomerase,

346 which reveals a unique gating mechanism for substrate specificity, due to the conformational
347 changes in the hydrophobic channel toward the active site. The length preference for C18 fatty
348 acids can thus be explained by the fixed distance between the FAD and the substrate
349 carboxylate (11 carbon atoms in the case of linoleic acid and linolenic acids) [108, 109].

350 5. Microbial cell factories for *t*10,*c*12-CLA production

351 The original bacterial CLA producers only recognize the free fatty acids as substrate
352 whereas high concentration of free fatty acids could inhibit the growth of bacteria. Therefore
353 those microbial CLA producers are not suitable for industrial purpose. With the genetic
354 determinants for CLA production available, genetic engineering and metabolic engineering
355 might be alternative to serve as microbial cell factories for commercial CLA production.

356 5.1 Bacteria

357 *Escherichia coli* is among the most commonly used host in genetic engineering.
358 Rosberg-Cody et al. demonstrated that *E. coli* cells carrying the construct co*PAI* gene
359 converted about 40% of LA [29]. Moreover, IPTG induction condition must be developed
360 otherwise linoleate isomerase expressed in *E. coli* would be no activitity [79]. Further studies
361 demonstrated that fed batch fermentation could be considered as an effective method to
362 improve the expression of PAI with significant activity. Except this typical C9 isomerase, C12
363 linoleate isomerase was also successfully hetero-expressed in *E.coli* [110]. All these trials
364 would provide an effective product on process of CLA for the medical and nutritional purposes.
365 However, the final concentration of CLA was limited by the resistance of *E. coli* in fatty acids.
366 PAI from *P. acnes* was cloned and successfully heterologeously expressed in *L. lactis* NZ9800
367 [29]. Approximately 50% of linoleic acid could be converted to *t*10,*c*12-CLA by the
368 recombinants, which could also significantly inhibit the growth of SW480 cancer cells.
369 Ingestion of recombinant *L. paracasei* NFBC 338 with PAI gene could lead to 4-fold increase in
370 *t*10,*c*12 CLA in adipose of mice and 2.5-fold increase in liver [78]. The cost for industrial
371 fermentation of *L. lactis* is expensive, which results in products without price advances.

372 5.2 Yeast

373 **Baker's yeast.** *Saccharomuces cerevisiae* is another well studied organism for metabolic
374 engineering. For example, it has been metabolically engineered to produce artemisinic acid
375 and amorpha-4,11-diene [110]. Expression of PAI genes in *S. cerevisiae* has also been

successful in recent years, in which the amount of CLA could be up to 57% of total free fatty acid [107]. *S. cerevisiae* seems to be a potential microbial cell factory for CLA production.

Oleaginous yeast. An oleaginous yeast, *Yarrowia lipolytica*, is naturally capable of accumulating lipids to levels exceeding half of dry cell weight (DCW), and with appropriate genetic modifications, such as abolishing the lipid turnover pathway, lipid accumulation could exceed 80 % of DCW [111]. *Y. lipolytica* could accumulate >90 % of neutral lipids in the triacylglycerols (TAG). These unique features of *Y. lipolytica*, together with the availability of genetic tools, have already attracted great interest in the usage for bio-oil production. With a series of genetic modification (overexpression and knockout), the final engineered yeast lipid comprises EPA at 56.6% by weight, which was the highest among known EPA sources [112]. Our research firstly reported the production of *t*10,*c*12-CLA by recombinant *Y. lipoltica* with PAI overexpression. It is notable that with multi-copy integration the yield of CLA is appropriately 30 times in yeast carrying the codon-optimized gene than that carrying the native gene, which is approximately 7.0 mg/l for the former strains. Lately the amount of *t*10,*c*12-CLA in *Y. lipolytica* Polh was increased to the level of up to 10% by co-expressing delta 12-desaturase gene together with the co-PAI multi-copy integration [30]. In addition, permeabilization of the *Y. lipolytica* by freeze/thawing has also been identified to significantly increase the amount of *t*10,*c*12-CLA up to 15.6 g/l and also to remain the extracellular production of *t*10,*c*12-conjugated linoleic acid above 10 g/l, and now with LA adding the CLA yield is around 22 g/l (Fig. 5) [113]. A recent research by other researchers tried to produce CLA within *Y. lipolytica* as well, and the final concentration of CLA is approximately 302 mg/l [31]. Hence, *Y. lipolytica* could be considered as the suitable microbial cell factory for CLA production via bioengineering. Additionally, several technologies, including various fermentation configurations, have been already used for single-cell oil production by strains of *Y. lipolytica* grown on various agro-industrial by-products or waste, which could be perfect sources for sole CLA isomer manufacture.

5.3 Oleaginous fungus

An oleaginous fungus, *Mortierella alpina*, has been used for commercial production of arachidonic acid (AA), in which fatty acids could accumulate up to approximately 50% of DCW. The relative abundance and proportion of its PUFAs makes *M. alpina* a perfect source of some

specific nutritional supplements. Moreover, the genome of *M. alpina* has been fully characterized [114] and an *Agrobacterium tumefaciens*-mediated transformation (ATMT) system has been established successfully [115]. As *M. alpina* could accumulate a high content of PUFAs, including linoleic acid (~10% of total fatty acids), it could be a valuable organism for CLA production. The incorporation of *t*10,*c*12 CLA into the PUFAs of *M. alpina* via PAI conversion, if achieved at a high level, would significantly increase the commercial value of *M. alpina*. We tried heterologously expression of codon-optimized PAI gene in *M. alpina* via ATMA system [116]. The amount of *t*10,*c*12-CLA increased up to 1.2 mg/l and reached up to 29 mg/l when acyl CoA synthetase inhibitor was added. With free LA and Triacsin C addition together, the final CLA reached 4.05% TFA (Fig 7). Heterologous expression of PAI in *M. alpina* could be considered as another practicable way for industrial production of CLA.

6. Perspectives

6.1 Further mechanistic exploration for lactic acid bacteria.

Recently, the system control of CLA production among lactic acid bacteria could be divided into two groups, group I and group II. Group I includes three genes that is *mcra, cla-dh and cla-dc* and the synthesis pathway has been elucidated. Group II (mainly among bacteria strains) has been identified to be a new system for CLA production, in which 10-hydroxy-octadecenic acid is not one of the intermediates, but the intricate mechanism is not clear. Thus, more efforts should focus on exploring this new system, for the purpose of broadening our knowledge about CLA production among lactic acid bacteria and providing more synthetic routes of CLA for manufacturers.

6.2 Microbial cell factories development and manufacturing of CLA.

Commercial CLA are produced through chemical process or extract from plant seeds, which result in mixtures of CLA in the final products. A number of bacteria have been reported to produce unique CLA effectively, which could be potential microbial cell factories for CLA production, whereas fermentation cost and low concentration of CLA, those high CLA producers might not be ideal microbial cell factories. Recombinant technology seems to be an effective alternative. For oleaginous microorganisms, *Y. Lipolytica* and *M. alpina*, have been successfully introduced for 10,12-CLA production, which is much close to industry purpose. Later, fermentation condition should be optimized to meet the industrial needs. The main

9,11-CLA producers include *B. fibrisolvens*, *C. sporogenes, L. plantarum, Bifidobacterium.* Among all these strains, only the genes among *L. plantarum* responsible for the CLA production were clear, including *mcra*, *cla-dh* and *cla-dc*. In the future, many trials should be carried out to express these three genes in some model engineering strains, such as *Y. lipolytica* and *M. alpina.*

Acknowledgement

This research was supported by the National Natural Science Foundation of China (Nos. 31571810, 31722041, 31530056), the National Natural Science Foundation of Jiangsu Province (No. BK20150141), the Fundamental Research Funds for the Central Universities (Nos. JUSRP51702A, JUSRP11733) and the Jiangsu Province "Collaborative Innovation Center for Food Safety and Quality Control".

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Figure captions

Fig. 1 Proposed model for the isomerization of linoleic acid by linoleic acid isomerase in rumen microorganisms.

An electrophile (E), at the active site, interacts with one of the substrate double bonds and two basic centers also interact with the substrate. One of the basic centers (B) is hydrogen bonded to the carboxyl group of the substrate and the other (B-H) serves as the donor of the hydrogen added at C-13 (From Kepler et al., 1971).

Fig. 2 Different conformation of domain 4 observed in MCRA monomers from *L. acidophilus*.

(A) A superposition of two MCRA protomers differing in the conformation of domain 4. Domain 1, 2, 3 and 4 of the apo MCRA protomer (symmetric protomer) are coloured marine, light green, red and yellow, respectively. The LA-MCRA protomer (asymmetric protomer) exhibiting a different conformation of domain 4 is coloured grey. The missing residues of domain 4 (556-574) are marked as a grey dashed line and the remaining traceable fragment of domain 4. (B) Superposition of apo LAH and LA–LAH dimers viewed along the dimerization axis. Domains 1, 2, 3 and 4 of apo LAH are coloured marine, light green, red and yellow/wheat, respectively. The protomomers of LA–LAH are coloured in grey and dark grey for the symmetric and the asymmetric protomer, respectively. Residue Lys575 is labelled in both the symmetric protomer (apo LAH, loop region) and the asymmetric protomer. (C) Linoleic acid bound at the entrance to the MCRA substrate and the electron density observed at the substrate-entrance channel. (D) Linoleic acid bound at the entrance to the LAH substrate channel. (From Volkov et al., 2013)

Fig. 3 CLA production pathway and hydroxyl fatty acid generation in lactobacilli

10-HOE was one of the intermediates during CLA production in *L. plantarum* and *L. acidophilus*. 10-LAH, DH and DC were the enzymes for CLA production in *L. plantarum* AKU1009a and *L. plantarum* ZS2058, while 10-LAH and enolase were the enzymes for CLA production in *L. plantarum* ATCC 8014. 10-LAH: linoleate 10-hydratase, 13-LAH: linoleate 13-hydratase, DH: short-chain dehydrogenase/oxidoreductase; DC: acetoacetate decarboxylase. 13-HOE: 13-hydroxy-*cis*-9-octadecenoic acid; 10-HOE:

10-hydroxy-*cis*-12-octadecenoic acid; 10,13-diHOA: 10,13-hydroxy-octadecanoic acid.

Fig. 4 Structure-based isomerization mechanism of linoleic acid to 10, 12-CLA

(A) Architecture of PAI. The FAD-binding domain 1 is colored in magenta, domain 2 in red, and domain 3 in blue. FAD and polyethylene glycerol (PEG) 400 are shown as stick models. (B) The surface potential of PAI. (C) The molecular surface (blue) of part of the PEG400 molecule bound to PAI in absence of substrate/product. (D) Conformational changes in active site associated with PEG400 binding reveal the gating mechanism. (From Liavonchanka et al., 2006). (E) Structure-based isomerization mechanism of linoleic acid to *t*10,*c*12-CLA

Fig. 5 CLA production strategy in *Yarrowia lipolytica* and in *Mortierella alpina*

DHAP: dihydroxyacetone phosphate; G3P: glycerol-3-phosphate; LPA: lysophosphatidic acid PA: phosphatidic acid; DAG: diacylglycerol; TAG: triacylglycerol; FFA: free fatty acids.

G3PD: G3P dehydrogenase; *SCT*: glycerol-3-phosphate acyltransferase; *PAP*: PA phosphohydrolase; *DGA*: acyl-CoA:DAG acyltransferase; *TGL*: triacylglycerol lipase; *ACOT*: Acyl-CoA thioesterase; *FAA*: fatty acyl-CoA synthetase; *FAS*: fatty acid synthase; *PAI*: polyunsaturated fatty acid isomerase. (Those genes and approach for enhancing CLA production were highlighted in red arrows and cycles, in which the solid ones have been done. Those genes and pathway for reducing CLA accumulation were highlighted in green arrows.)

Fig. 1

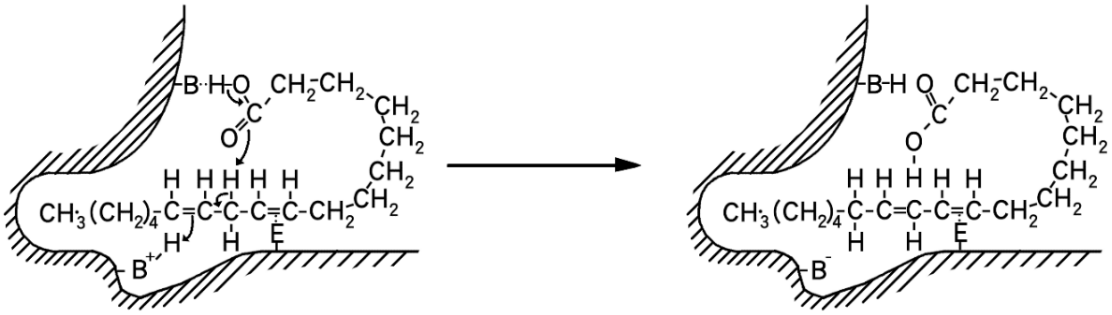
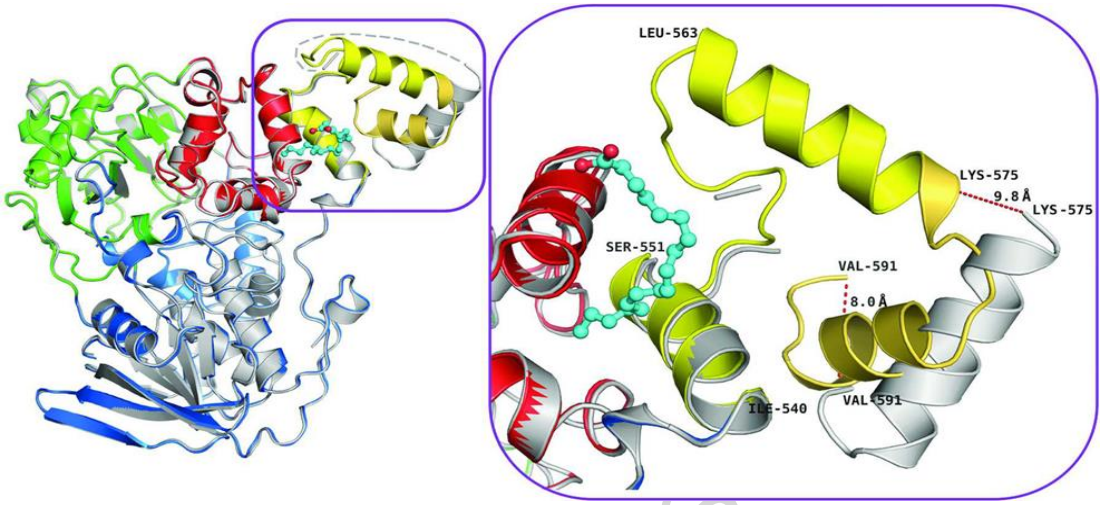


Fig. 2

(A)

(B)



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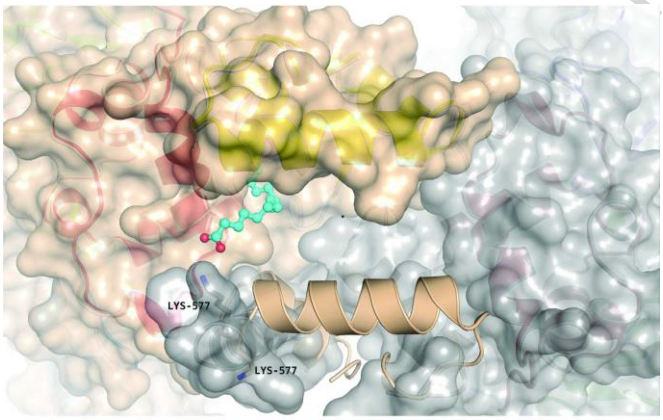

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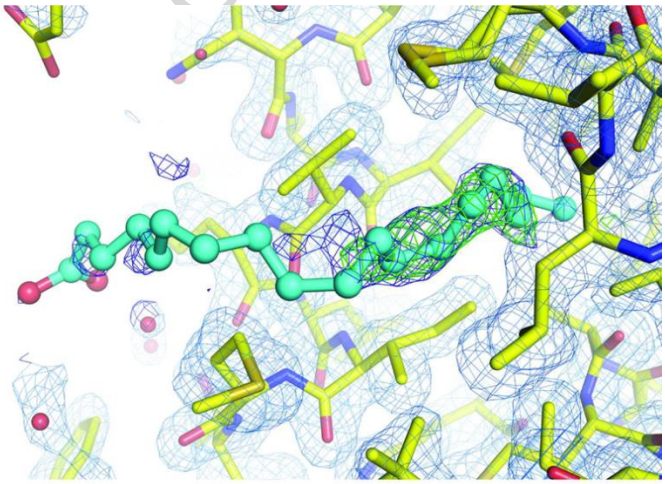

Fig. 3

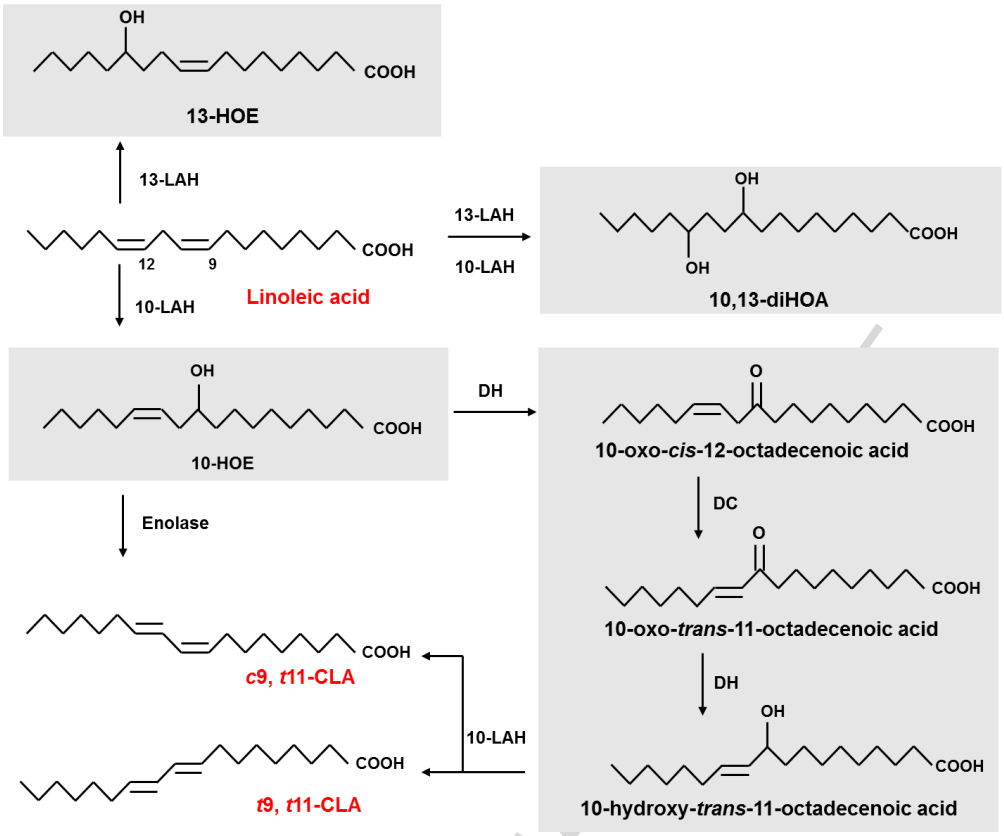

Fig. 4

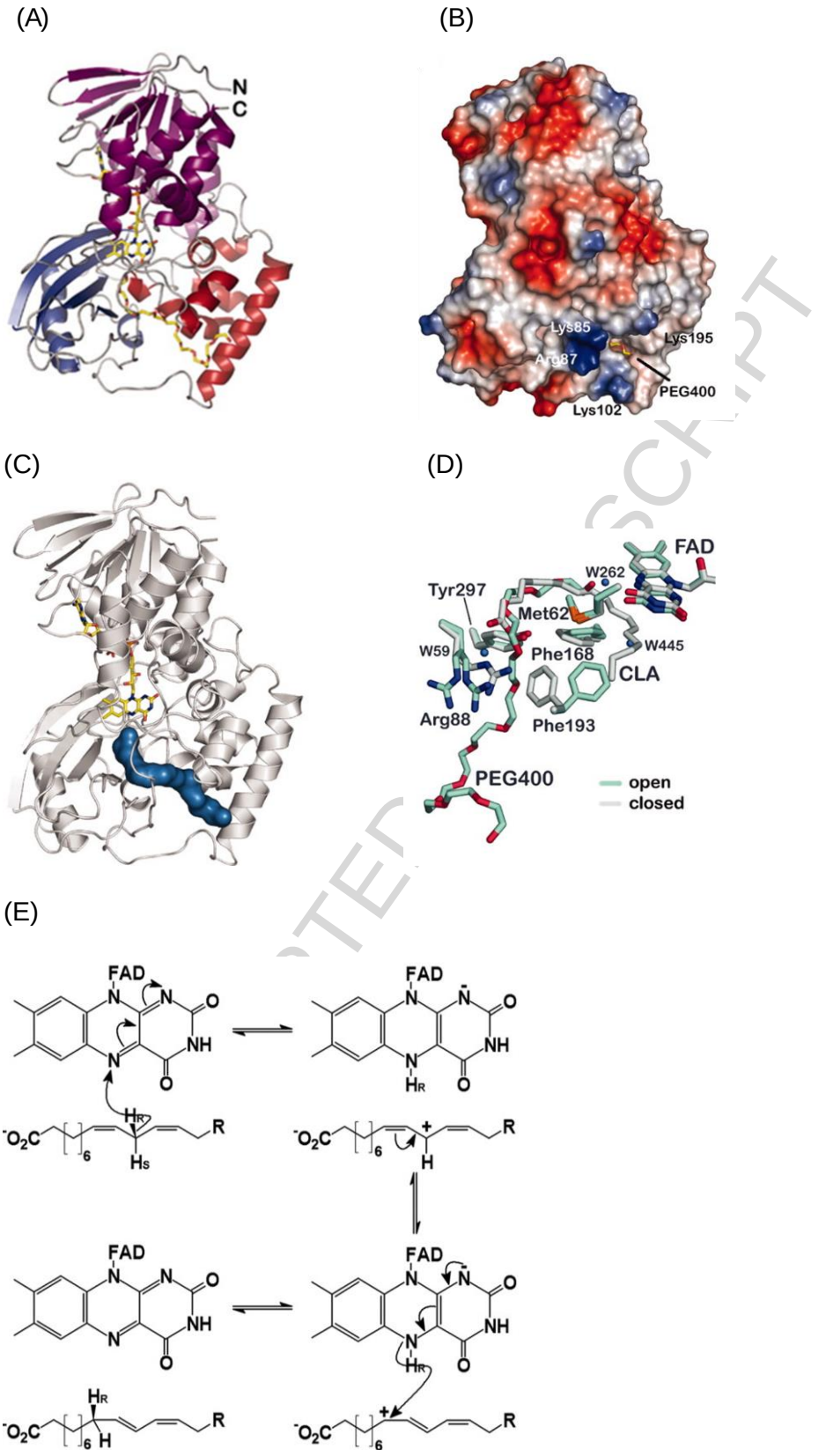

Fig. 5

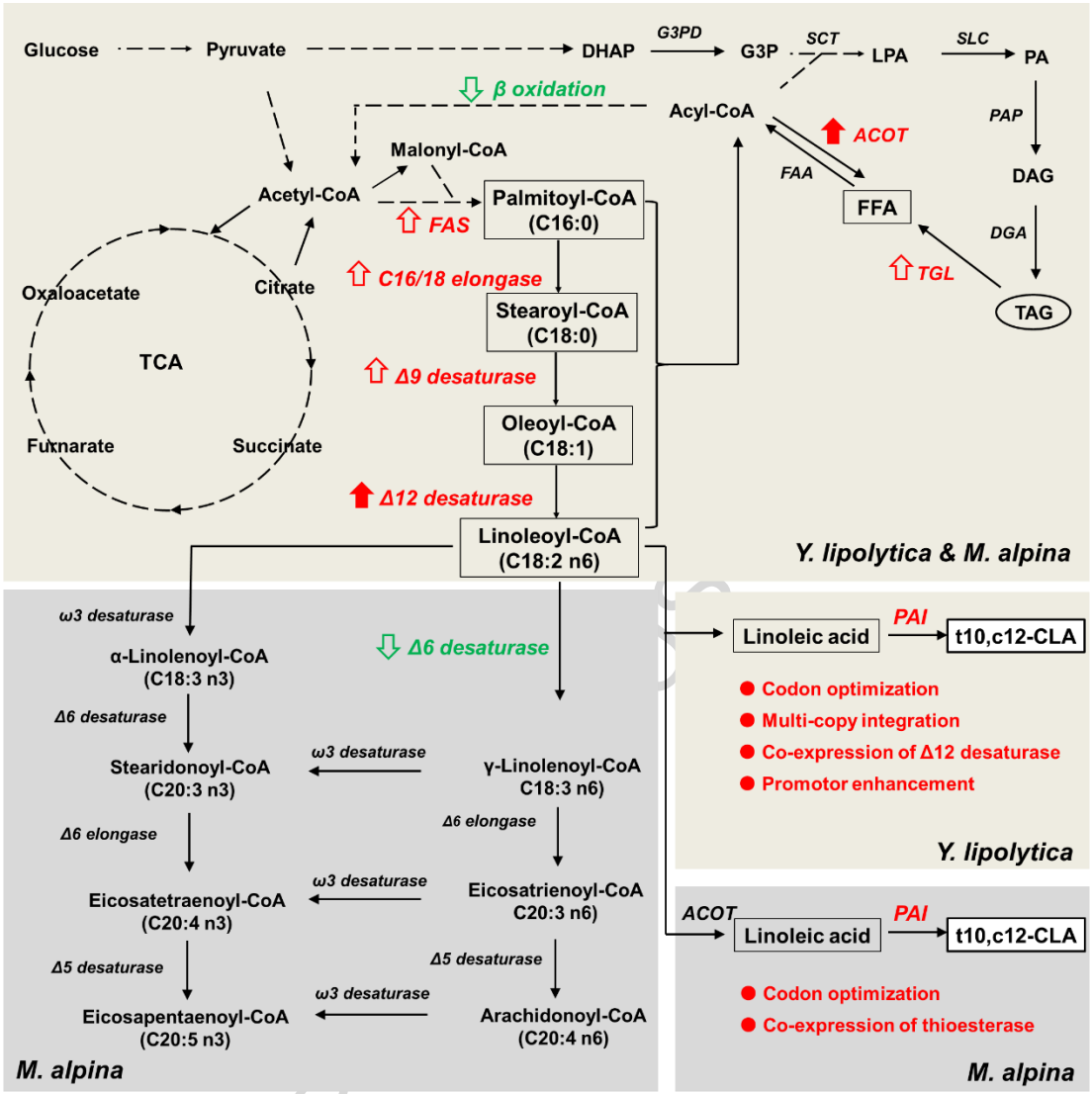

Table 1 High CLA-producing strains

Species	Strain No.	Substrate (g/l)	Catalyst	CLA				Ref.
				Total CLA (g/l)	Conversion (%)	9,11-CLA (%)	10,12-CLA (%)	
Butyrivibrio fibrisolvens	A38	LA (0.10)	washed cells	0.080	80.0	95	5	[37]
Lactobacillus acidophilus	1.184	LA (2.00)	washed cells	1.728	86.4			[117]
	CRL 730	LA (0.20)	growing cells	0.048	23.8			[71]
	Q42	LA (0.20)	growing cells	0.040	20.0			[71]
	L1	LA (0.20)	growing cells	0.131	65.5	90	10	[21]
	O16	LA (0.20)	growing cells	0.061	30.5	91	9	[21]
	AKU 1137	LA (4.00)	washed cells	1.500	37.5	100	0	[19]
	AKU 1137	LA (5.00)	growing cells	4.900	98.0	100	0	[28]
Lactobacillus brevis	IAM 1082	LA (4.00)	washed cells	0.550	13.8	100	0	[19]
Lactobacillus casei	CRL 431	LA (0.20)	growing cells	0.072	35.9			[71]
	E10	LA (0.20)	growing cells	0.080	40.1	91	9	[21]
	E5	LA (0.20)	growing cells	0.111	55.5	88	12	[21]
Lactobacillus plantarum	JCM 1551	LA (4.00)	washed cells	2.020	50.5	100	0	[19]
	JCM 1551	castor oil (5.00)	growing cells	2.700	54.0	100	0	[44]
	ZS2058	LA (0.55)	growing cells	0.300	54.5	56	3	[45]
	lp 15	LA (0.10)	growing cells	0.026	26.1	76	24	[118]
	PL62	LA (1.00)	crude enzyme	2.65*		47	53	[119]
	NCUL005	LA (2.28)	growing cells	0.602	26.4	32	69	[82]
Lactobacillus pentosus	C14	LA (0.20)	growing cells	0.069	34.5			[71]
Lactobacillus reuteri	ATCC55739	LA (0.55)	growing cells	0.350	63.6	97	3	[45]
	ATCC55739	LA (0.90)	growing cells	0.300	33.3	59	41	[18]
Bifidobacterium animalis	Bb12	LA (0.56)	growing cells	0.170	30.4	31	1	[45]
Bifidobacterium breve	NCFB2257	LA (0.55)	growing cells	0.231	42.0	99	1	[65]
	NCFB 2258	LA (0.55)	growing cells	0.398	72.4	100	0	[65]
	NCFB11815	LA (0.55)	growing cells	0.215	39.1	99	1	[65]
	NCFB8815	LA (0.55)	growing cells	0.242	44.0	99	1	[65]
	NCFB 8807	LA (0.55)	growing cells	0.128	23.3	99	1	[65]
	LMC 520	LA (0.56)	growing cells	0.400	71.4	95	5	[23]
Bifidobacterium bifidum	CRL 1399	LA (0.20)	growing cells	0.050	24.8			[71]
Bifidobacterium lactis	Bb12	LA (0.55)	growing cells	0.170	30.9	98	2	[65]
Enterococcus faecium	M74	soy oil (10) [b]	growing cells	0.73[a]		100	0	[77]
Megasphaera elsdenii	YJ-4	LA (0.02)	growing cells	0.007*		15	85	[80]
Pediococcus acidilactici	AKU1059	LA (4.00)	washed cells	1.400	35.0	100	0	[19]
Propinibacterium acnes	No.27	LA (0.02)	growing cells	0.017	85.0	0	100	[26]
Propinibacterium	P-6 Wiesby	LA (0.75)	growing cells	0.265	35.3	93		[73]

freudenreichii	9093	LA (0.50)	growing cells	0.111	22.2	90		[73]
	ATCC 6207	LA (0.10)	growing cells	0.023	23.2	75		[73]
Propinibacterium	56	soy oil (10) [b]	growing cells	1.09[a]		83	17	[77]
freudenreichii subsp.	51	soy oil (10) [b]	growing cells	1.65[a]		85	15	[77]
shermanii	23	soy oil (10) [b]	growing cells	0.81[a]		75	25	[77]
Streptreptococcus	CRL 728	LA (0.20)	growing cells	0.068	33.9			[71]
thermophilus	CRL728	LA (0.20)	growing cells*	0.105	52.5			[71]

* represents the reaction occurs in the skim milk;
[a] represent the results is expressed as mg CLA content per gram lipid; [b] means that soy oil is used in the hydrolyzed form.